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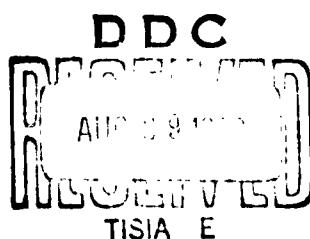
PRIMER ACTIVITY OF THYMUS DNA FRACTIONATED BY
ECTEOLA COLUMN CHROMATOGRAPHY

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ABSTRACT

The DNA-primer activity of Ecteola fractionated calf- and rat-thymus DNA samples was determined by a DNA-polymerase assay system. All DNA samples assayed, heated or unheated, and fractionated or unfractionated, showed some degree of DNA-primer activity.

The chromatographic profiles of heated DNA were different from those of unheated DNA. Phenol and p-aminosalicylate, used in the preparation of DNA, did not alter the patterns of DNA obtained by Ecteola chromatography. Sephadex chromatography was found to be a rapid and effective method to deionize DNA solutions.

DNA-primer activity was increased by an ammonium hydroxide gradient. At 4°, treatment of DNA by phenol and p-aminosalicylate, Ecteola column chromatography with a salt gradient, Sephadex column chromatography with deionized distilled water to desalt DNA, and lyophilization did not alter DNA-primer activity.

The results indicate that DNA-primer activity, per se, is not specifically associated with one particular fraction of DNA.

SUMMARY

The Problem:

The unique property of DNA to act as a primer for its own synthesis by the DNA-polymerase enzyme system is well established. In connection with the general question of the role of DNA-primer in cell division in vivo, the question arises whether there exists in rapidly dividing tissues a specific fraction of DNA with primer activity. The problem was to separate thymus DNA into fractions that demonstrated higher and lower DNA-primer activities than did the original starting material.

The Findings

Calf- and rat-thymus DNA were found to be heterogeneous and could be fractionated into separate chromatographic peaks by Ecteola column chromatography.

The chromatographic profiles of heated DNA were different from those of unheated DNA. Phenol and p-aminosalicylate, used in the preparation of DNA, did not alter the patterns of DNA obtained by Ecteola chromatography. Sephadex chromatography was found to be a rapid and effective method to deionize DNA solutions.

DNA-primer activity was increased by an ammonium hydroxide gradient. At 4°, treatment of DNA by phenol and p-aminosalicylate, Ecteola column chromatography with a salt gradient, Sephadex column chromatography with

deionized distilled water to desalt DNA, and lyophilization did not alter DNA-primer activity.

Primer assay of the DNA fractions indicated that primer DNA was not separated from nonprimer DNA.

INTRODUCTION

The classical work of Kornberg, et al.¹, demonstrated a requirement for DNA as primer in the enzymatic synthesis of DNA by the polymerase system. Later, a marked increase in priming activity of DNA after heating was observed ^{2,3}. Heated versus unheated DNA samples have been differentiated also by characteristic chromatographic patterns ^{4,5}. More recently, an enzyme-induced increase in priming activity of various "native" DNA samples in the *E. coli* DNA-polymerase system has been reported ⁵.

In this connection, the question arises whether DNA can be fractionated into primer and nonprimer DNA, and whether all DNA molecules have priming activity. Therefore, we investigated the DNA-primer activity associated with Ecteola column chromatographic fractions of unheated versus heated calf- and rat-thymus DNA samples. In order to accomplish this, DNA was prepared by a phenol extraction method, fractionated with an Ecteola column, desalts with Sephadex, lyophilized, and assayed for DNA-primer activity in a DNA-polymerase system.

METHODS AND MATERIALS

Preparation of DNA samples. Worthington Calf-thymus DNA (67 mg) was dissolved in deionized distilled water (93.3 ml) by stirring with a magnetic stirrer for 22 hours at 4°. Tris-phosphate buffer (6.7 ml, 0.5 M, pH 7.3) was then added and a portion (50 ml) was used for treatment with phenol to determine if there would be an effect of phenol on DNA-primer activity of DNA thus isolated. This portion was mixed with an equal volume of 90% w/w freshly-distilled phenol. The resulting solution was stirred for one hour at 4° and centrifuged at 20,000 rpm in a Spinco Model L centrifuge with a swinging bucket head (SW-25.1) for 30 min at 0°. The upper aqueous layer was removed with the aid of a tube-cutter and mixed with an equal volume of 2-ethoxy ethanol (ethyl cellosolve, mono-ethyl ether of ethylene glycol) at 0° to precipitate DNA in a fibrous form. The fibers were wrapped around a glass rod in a manner in which most of the mother liquor was squeezed out of the fibers, removed from the flask, and dissolved in deionized distilled water (25 ml). Enough tris-phosphate buffer (0.5 M, pH 7.3) was then added to effect a dilute DNA solution (in 0.03 M buffer) such that the A_{260} was approximately equal to the calf-thymus DNA aliquot not treated with phenol.

Rat-thymus DNA was prepared by the method of Kirby⁷ and also by a simplified modification of it. The thymus glands of 5-1/2 week old female Sprague-Dawley rats were removed and homogenized at 0° in sucrose-

tris-chloride buffer (sucrose, 0.23 M; tris, 11 mM; pH 7.7) and centrifuged (105,000 \times g, 1 hr, 0°). The resultant supernatant fluid, containing DNA-polymerase, was stored at -196°. The insoluble nucleoprotein pellet obtained (12 g) was stored at -20° before extracting the DNA. It was homogenized with 6% w/v sodium p-aminosalicylate (15 vol) and then stirred with 90% w/w phenol (15 vol) for one hour at 4°. The mixture was centrifuged and the DNA was recovered as described above for the DNA treated with phenol. At this point a modification of the method was introduced, in that the DNA was recovered without further treatment. The DNA fibers obtained in each case were dissolved in deionized water (100 ml) at 4°. Buffer was added, the A_{260} was determined, and the appropriate dilution made as above.

A portion of each of the above samples was heated in a boiling water bath (100°) for 7 min and cooled quickly. The A_{260} of each sample was determined in tris-phosphate buffer (0.03 M, pH 7.3), and the DNA samples were stored at -20° for later use.

Ecteola column chromatography. The method used was an extensive modification of that of Rosenkranz and Bendich⁴. A linear gradient was set up in the cold room (4°) where two graduated cylinders (250 ml) were connected at their base so that the fluid levels in each cylinder remained equal during elution. The eluting solvent in one cylinder was mixed with a magnetic stirrer before being conducted from the base of that container to the column by polyethylene tubing. Ecteola cellulose anion exchanger (type 20, 0.4 meq N/g), purchased from Brown Co., Berlin,

N. H., was successively washed with water, 1 N NaOH, water, and buffer (0.05 M NaCl, 0.001 M phosphate, pH 7.0) before use. The column (1.0 g Ecteola, 1. cm x 6.5 cm) was equipped with a constant level siphon to prevent the column from going dry when the eluting solvent was exhausted. The amount of DNA sample (about 4 mg in 6.0 ml) put on the column was determined in terms of DNA-units ($A_{260} \times \text{vol}$). The flow-rate was about 0.5 ml per min and the fractions (60 drops per tube, about 6 to 7 ml) were collected with an automatic fraction collector. The method of linear gradient elution consisted of two steps: I) A salt gradient from 0.05 M NaCl to 2.0 M NaCl (50 ml each, buffered with 0.001 M phosphate, pH 7.0); and II) An NH_4OH gradient to 2.0 N NH_4OH in 2.0 M NaCl (150 ml each). The volume and A_{260} of the fractions collected were measured before storing them at -20°. The same column was used repeatedly by washing the Ecteola with 1 N NaOH, water, and buffer before applying a new sample.

Sephadex column chromatography. The general methods used were modifications of those of other workers^{8,9}. Sephadex gel (G-25, coarse) was purchased from Pharmacia, Uppsala, Sweden, and was washed repeatedly with deionized distilled water to remove "fines". Two different columns were prepared in a similar manner: one (2.5 cm x 40 cm) was prepared with 30 g and, the other (3.5 cm x 30 cm) with 60 g quantities. They were equipped with constant level siphons to prevent the Sephadex from going dry when the eluting solvent was exhausted. The fractions eluted from the Ecteola columns and saved at -20° were thawed and put on the

Sephadex column in the cold room (4°) for chromatography in order to separate the DNA from NaCl and NH_4OH . Deionized distilled water was used as the eluting solvent. The flow-rate was 1-2 ml per min and the fraction size collected with an automatic fraction collector was 6-7 ml per tube. The $A_{260} \times$ vol, electrical resistance, and pH of the fractions were determined at 25° . Appropriate tubes selected for lyophilization were combined and the determinations were made as above, in order to prepare the DNA for primer assay. The samples thus prepared were stored at -20° for later use. The Sephadex columns were used repeatedly after thorough elution of retained NaCl and NH_4OH with an excess of water.

DNA-primer assay. The incorporation of (3H) thymidine into newly synthesized DNA was determined, in triplicate, by the method of Walwick and Main¹⁰. The assay mixture (total volume of 500 μ l) contained: (3H) thymidine (6 μ M, specific activity 3.00 c per mmole); deoxynucleoside 5'-monophosphates of adenine, cytosine, and guanine (50 μ M each); $MgCl_2$ (5.0 mM); KCl (45 mM); tris-ATP (6.0 μ M); crude DNA-polymerase supernatant fluid (250 μ l, 2.0 mg protein) and varying amounts of DNA, as DNA primer, per 150 μ l tris-phosphate buffer (0.03 M, pH 7.3). The mixture was incubated at 37° for 1 hour for the assay.

Determination of DNA. An indole method¹¹ was used to determine the amount of DNA assayed for DNA-primer activity.

RESULTS

Ecteola column chromatography. Calf- and rat-thymus DNA, prepared as described above, were chromatographed with an Ecteola column (Figs. 1-4). The recovery of DNA-units from the column was 92-107 %. Except for one fraction (tube nos. 1-2 of Figs. 2 and 3) identified as phenol, and another (tube nos. 4-7 of Figs. 2 and 3) identified as p-aminosalicylate, the elution patterns of untreated calf-thymus DNA and that treated with phenol and p-aminosalicylate were similar. The chromatographic profiles of heated DNA were different from those of unheated DNA (compare Figs. 1A-4A with Figs. 1B-4B), conforming to the observations of Rosenkranz and Bendich⁵.

DNA prepared by the unmodified Kirby⁷ procedure versus the modified method yielded different chromatographic patterns (compare Figs. 3 and 4). In addition, fractions obtained by the unmodified method⁷ contained no phenol or p-aminosalicylate and the amount of the DNA fraction eluted in the salt gradient was considerably reduced.

Deionization by Sephadex column chromatography. The major DNA fractions from the Ecteola column were deionized by Sephadex chromatography. The recovery of DNA-units varied between 84-102%. Typical examples with the two different sizes of columns are shown in Fig. 5. The concentration of NaCl found in the DNA fractions was only 0.1 mM as determined by electrical resistance of the solutions. In addition, the neutral pH of the DNA fractions demonstrated the absence of NH₄OH (Fig. 5).

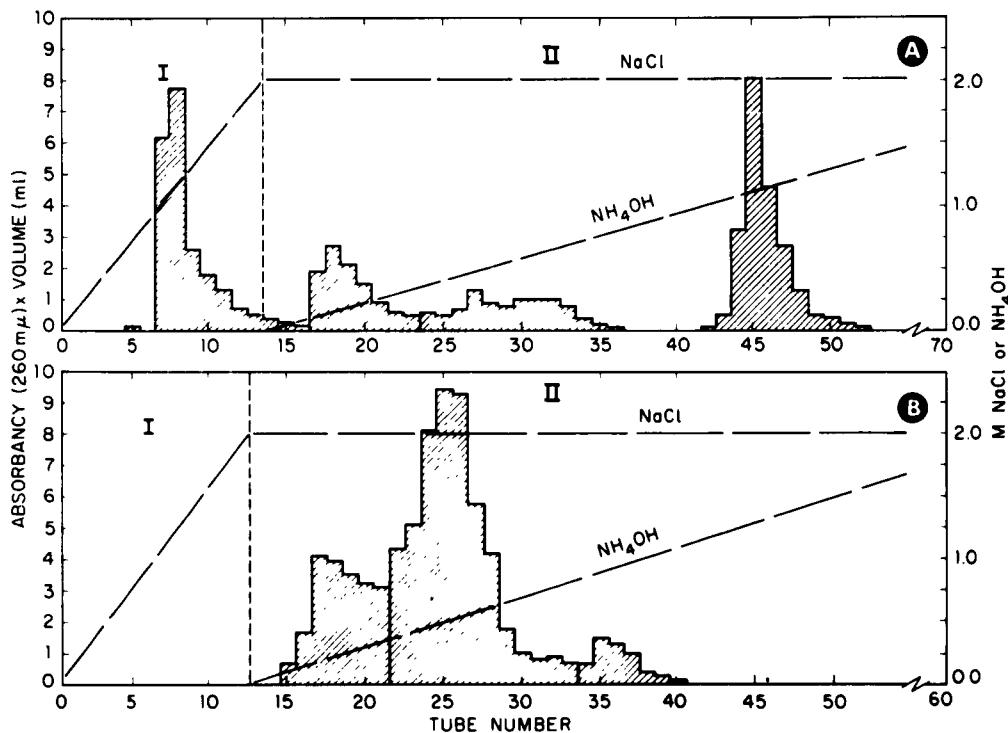


Fig. 1A Ecteola column chromatographic pattern of 67 DNA-units ($A_{260} \times \text{vol}$) of unheated calf-thymus DNA. The elution pattern in area I was the result of a linear salt gradient (0.05 M NaCl to 2.0 M NaCl) at pH 7.0, and in area II from a linear NH₄OH gradient (0.0 to 2.0 N NH₄OH) with constant salt concentration (2.0 M NaCl). Total recovery was 92%. Other details are presented in the methods and materials section.

Fig. 1B Same legend as in Fig. 1A except that the calf-thymus DNA was heated (100°, 7 min) prior to chromatography and 81 DNA-units were put on the column. Total recovery was 94%.

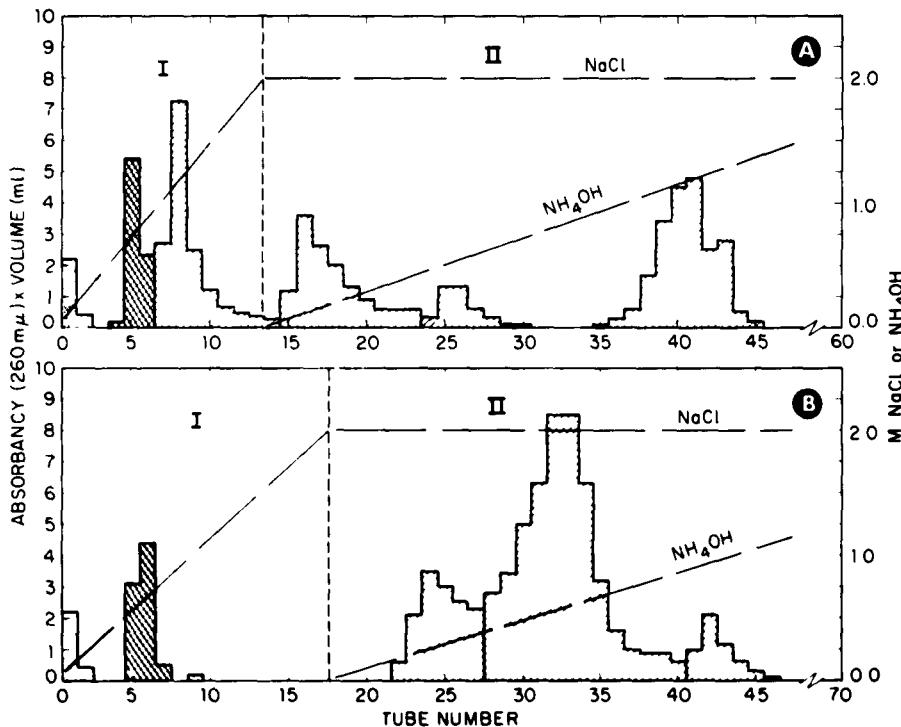


Fig. 2A Ecteola column chromatographic pattern of 67 DNA-units of unheated calf-thymus DNA which had been previously treated by a modified phenol extraction method. The elution pattern was obtained as described in the legend to Fig. 1. Tube nos. 1-2 and 4-6 represent phenol and p-aminosalicylate respectively. Total recovery was 95%.

Fig. 2B Same legend as in Fig. 2A except that the calf-thymus DNA sample was heated prior to chromatography and 78 DNA-units were put on the column. Total recovery was 100%.

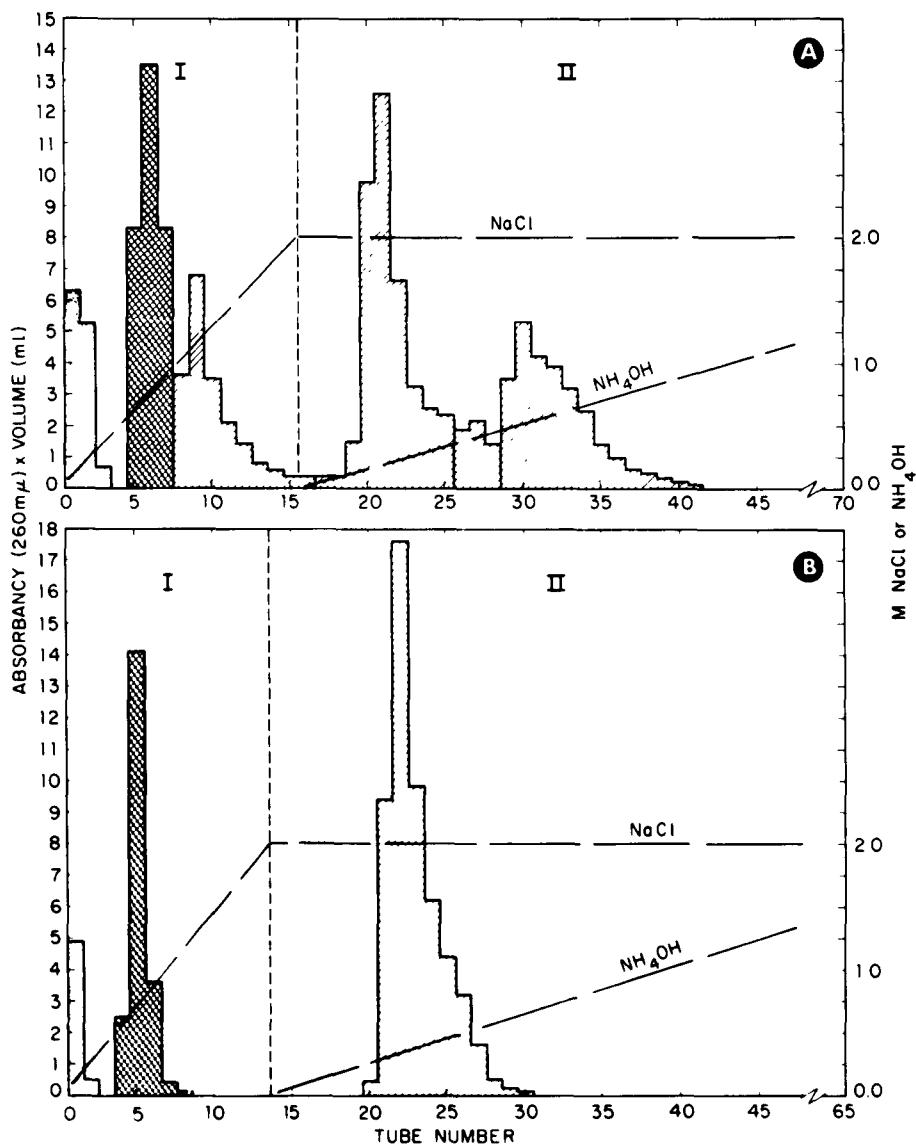


Fig. 3A Ecteola column chromatographic pattern of 136 DNA-units of unheated rat-thymus DNA obtained by a modified phenol extraction method. The elution pattern was obtained as described in the legend to Fig. 1. Tube nos. 1-3 and 4-7 represent phenol and p-aminosalicylate respectively. Total recovery was 107%.

Fig. 3B Same legend as in Fig. 3A except that the rat-thymus DNA sample was heated prior to chromatography and 77 DNA-units were put on the column. Total recovery was 100%.

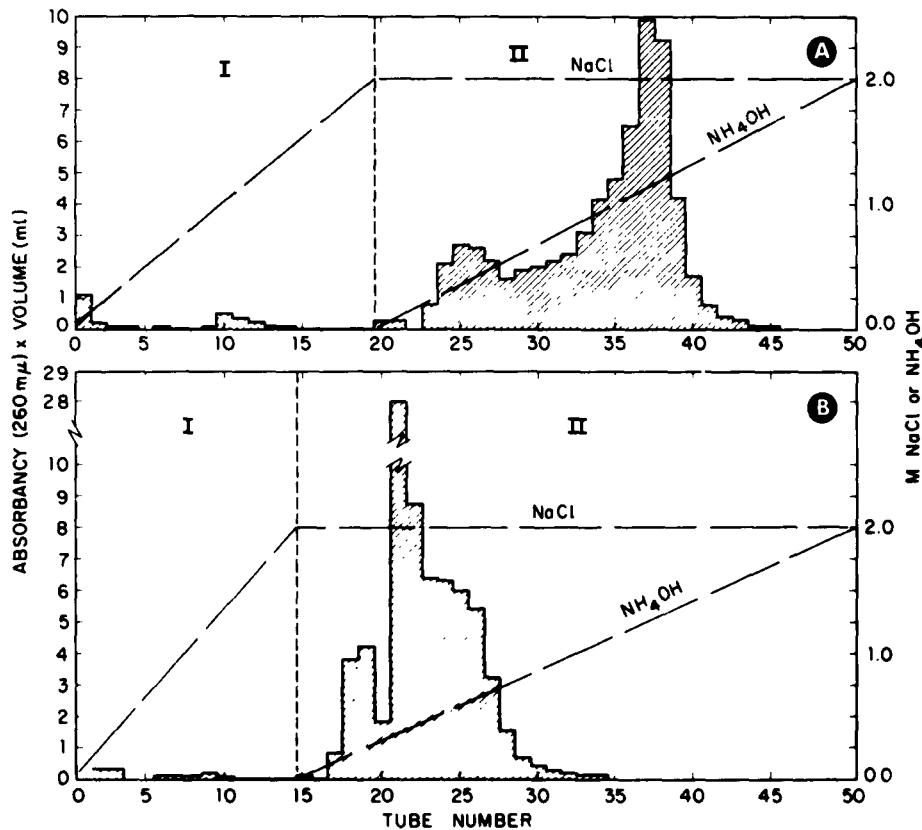


Fig. 4A Ecteola column chromatographic pattern of 65 DNA-units of unheated rat-thymus DNA obtained by the unmodified phenol method.⁷ The elution pattern was obtained as described in the legend of Fig. 1. **Fig. 4B** Same legend as in Fig. 4A except that the rat-thymus DNA sample was heated prior to chromatography and 76 DNA-units were put on the column.

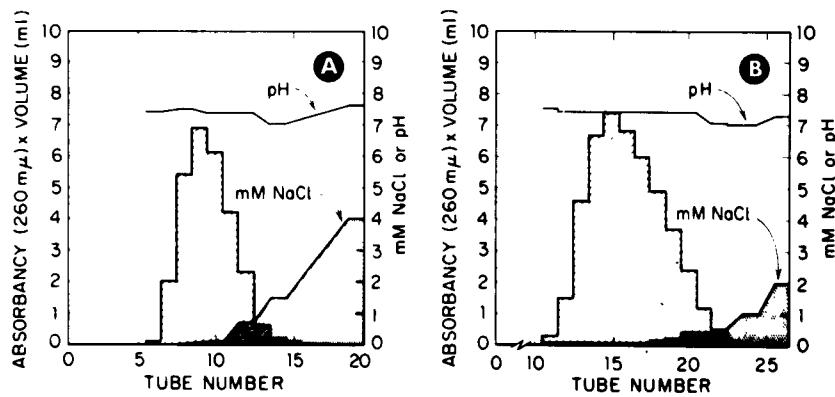


Fig. 5A Sephadex column (30 g G-25, 2.5 cm x 40 cm) chromatographic elution curves of an Ecteola column fraction of unheated rat-thymus DNA sample (tube nos. 20-23 of Fig. 3A, 28.6 DNA-units) at 4°. The curves were obtained by using deionized distilled water as eluting solvent and testing the fractions for A_{260} , volume, electrical resistance, and pH as indicated in the Methods section. The recovery was 98%.

Fig. 5B Same legend as Fig. 5A except that the sample chromatographed was heated rat-thymus DNA (tube nos. 21-26 of Fig. 3B, 45 DNA-units) and a different column was used (60 g G-25, 3.5 cm x 30 cm). The recovery was 102%.

Primer assay of unfractionated DNA. In the assay, primer activity was linear in the range of 10-30 μ g for unheated and heated DNA (Fig 6). This linearity was observed also with DNA isolated either with or without phenol and p-aminosalicylate. Thus, the use of these reagents for preparation of DNA did not increase the primer activity of DNA. The values for unheated rat-thymus DNA were found to fall on a straight-line plot different from that of calf thymus DNA. The activities of heated DNA samples showed marked increases (about 10 fold) above the corresponding activities of unheated samples.

Primer assay of fractionated DNA. In all cases, primer activity of the isolated DNA Ecteola fractions increased with increasing NH_4OH concentrations used for eluting the DNA fractions (for example, unheated calf-thymus DNA, tube nos. 44-48 and 39-43 of Table I). In parallel with this observation was the decrease in hyperchromic shift of the respective fractions (Table I). Primer activity of the last fraction of unheated DNA eluted with NH_4OH approached that of the heated DNA (Table I and Figure 6). Primer activity values for calf-thymus DNA isolated with and without phenol were approximately the same (for example, unheated calf-thymus DNA, tube nos. 8-10 and 7-9 of Table I), while that of unheated DNA eluted by the NaCl gradient did not change appreciably from the activity of the original DNA samples before fractionation (Table I and Figure 6).

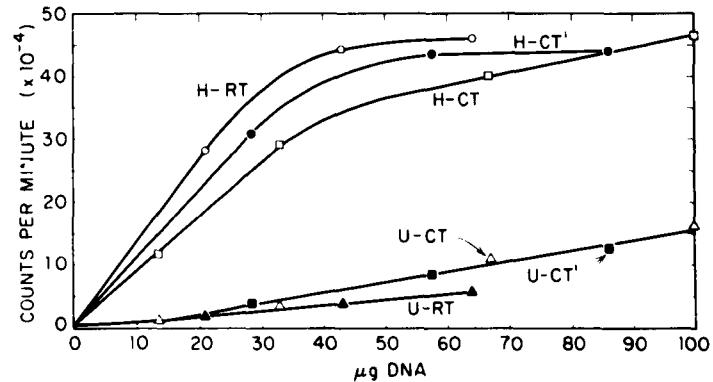


Fig. 6 The effect of various amounts of thymus DNA on the incorporation of (^3H) thymidine into DNA as a measure of DNA-primer activity in a DNA-polymerase assay system. The method of assay was performed as indicated in the methods section. The abbreviations are: U-CT and H-CT for unheated and heated calf-thymus DNA, U-CT' and H-CT' for unheated and heated calf-thymus DNA which had been previously treated with phenol, U-RT and H-RT for unheated and heated rat-thymus DNA obtained by a modified phenol extraction procedure as indicated in the methods section.

TABLE I
DNA-PRIMER ACTIVITY OF FRACTIONATED, DEIONIZED, AND LYOPHILIZED
THYMUS DNA

SAMPLE			DNA RECOVERED FOR ASSAY					
Source	Condition	Ecteola Fraction	Before Heating	After Heating	Hyperchromic Shift	DNA Assayed	DNA-primer Activity	
Calf	Unheated	tube #	A_{260}	A_{260}	%	μg	count, min $\times 10^{-3}$	
		8-10	0.100	0.130	30	10.0	8.7	
		17-20	0.100	0.130	30	9.0	15.4	
	Heated	44-48	0.125	0.140	12	10.4	102	
		17-21	0.125	0.135	8	9.5	106	
		22-28	0.115	0.130	9	9.5	111	
Thymus	Unheated	7-9	0.105	0.140	33	10.5	8.6	
		16-18	0.110	0.135	23	9.1	20.5	
	Heated	39-43	0.105	0.130	24	10.5	89.2	
		23-27	0.125	0.140	12	9.7	96.0	
After Phenol	Heated	28-35	0.095	0.110	16	11.0	102	
		8-11	0.100	0.135	35	10.2	12.5	
	Unheated	20-23	0.105	0.130	24	8.0	19.6	
		29-33	0.120	0.140	17	9.0	79.0	
Rat	Heated	21-26	0.120	0.130	8	8.5	91.6	
		blank	0.0	0.0	0	0.0	4.7	
Control							0.02	
Background								

DISCUSSION

The phenol method⁷ was modified to permit the assay of DNA as soon as possible in the isolation procedure in order to minimize the degradation or denaturation of DNA through subsequent methodological manipulations such as RNase treatment and repeated precipitations. The Ecteola column removed two impurities (phenol and p-aminosalicylate) from the DNA, thus isolated. Other criteria for degree of purity such as protein content of the DNA were not investigated. The unfractionated DNA samples assayed did contain these reagent impurities (phenol and p-aminosalicylate), but their presence did not affect the assay of unheated DNA for primer activity.

The gradient to 2 M NH₄OH was selected in order to elute the DNA quantitatively without the use of NaOH (cf.12). Gradient elution was preferred to stepwise elution to minimize the peaks associated with changes in eluting solvents^{5,6,13}. However, the use of NH₄OH gradient (in 2 M NaCl) produced an increase in primer activity of the DNA eluted by increasing concentrations of NH₄OH. It is evident that the exposure of DNA to NH₄OH increases its primer activity and thus introduces an artifact.

Work from this Laboratory¹⁰ has shown the importance of controlling cation concentration in the DNA-polymerase assay medium; therefore, it was necessary to remove salt from the DNA fractions obtained from chromatography before the assay. The use of Sephadex to deionize the

DNA fractions followed up by lyophilization to eliminate water did not complicate the interpretation of the data. It has also been reported¹⁴ that drying and rehydrating DNA does not uncoil its helical structure¹⁵ irreversibly.

If primer activity could be fractionated into one characteristic fraction, then it would be expected that the total activity of the unfractionated sample could be accounted for in such an active fraction. This was not found to be true in the present study. If the DNA could be fractionated into subfractions with different levels of primer activity it would seem logical to anticipate that the DNA recovered from the salt fraction would show different activity than the unfractionated DNA. However, the results from the salt gradient, show that this fraction had the same level of primer activity as did the unfractionated material. It is concluded, therefore, that under these conditions of DNA fractionation, primer activity was not concentrated in a particular DNA fraction.

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Naval Radiological Defense Laboratory
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by W.D. Skidmore, R.K. Main and L.J. Cole 12 June
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DNA-primer activity was increased by an ammonium hydroxide gradient. At 40°, treatment of DNA by phenol and p-aminosalicylate, Ecteola column chromatography with a salt gradient, Sephadex column chromatography with deionized distilled water to desalt DNA, and lyophilization did not alter DNA-primer activity.
The results indicate that DNA-primer activity, per se, is not specifically associated with one particular fraction of DNA.

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3. Fractionation.
4. Thymus.
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II. Main, R. K.
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